Pathogenesis of thrombosis

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The hemostatic process is a host defense mechanism to preserve the integrity of the closed high pressure circulatory system. This process must remain inactive but poised to minimize extravasation of blood from the vasculature following tissue injury. Given the complexity of the hemostatic mechanism, paradigms developed from biochemical and cell biological approaches have been revisited by studying thrombus formation in a live animal by intravital microscopy. Many of these paradigms have proven accurate, but others need to be reconsidered given the results of whole animal experiments.

The hemostatic process is a host defense mechanism—nature's effort to preserve the integrity of the closed high pressure circulatory system. This process must remain inactive but poised to immediately minimize extravasation of blood from the vasculature following tissue injury. Such a process must be activatable within seconds of injury. After this process is activated, it remains critical to contain thrombus formation so that it is localized to the site of injury and to modulate thrombus size to be proportionate to the injury. Thus, there is a balance between the pathways that initiate thrombus formation and the pathways that regulate or modulate thrombus formation.

Given the large cast of characters involved in thrombin generation during blood coagulation, platelet aggregation following activation, and regulatory pathways involved in thrombus formation, the primary approach to understanding these systems has been to isolate proteins or cells and study their function in vitro. Typically, a trace protein in plasma such as factor X was purified to homogeneity. The molecular basis of its activation to its enzyme form, factor Xa, was characterized biochemically. Its activation by the complex of factor IXa and factor VIIIa could be compared to its activation by factor VIIa/tissue factor. The role of calcium ions and phospholipid membranes in these reactions could be studied systematically by using biochemical techniques. Similarly, platelet function studies were performed with platelets purified away from other blood cells and away from plasma proteins. Receptors, such as $\alpha_{IIb}\beta_3$, could be characterized with regard to their interaction with fibrinogen in vitro. The number of receptors per platelet, characterization of the binding affinity of the ligand to the receptor, and identification of the activation state of the cell necessary to support ligand interaction could be defined. However, with a cast of characters approaching 100, it has required a leap of faith to predict the pathways of these reactions, their kinetics, and the biologic importance of specific reactions and interactions. Stated otherwise, what *can* happen has been determined by in vitro experiments. But these observations do not predict what *does* happen in vivo.

To address this question, we have developed a system for studying thrombus formation in a live mouse. This intravital imaging system, which has been amply described elsewhere,[1] allows for the near-simultaneous imaging of three separate fluorescent probes as well as a brightfield imaging to provide histologic context. Fluorescent probes can be attached to specific proteins, antibodies or cells, thus allowing their identification during thrombus formation. A transparent vascular window, either cremaster muscle or the mesentery, is studied in the anesthetized mouse. Thrombus formation is initiated either with a laser pulse to the vessel wall[2] or, alternatively, with the topical introduction of ferric chloride,[3] an agent that leads to denudation of the endothelium and the exposure of the subendothelial matrix. These methods of thrombus formation are, of course, artificial and only useful for developing experimental thrombi. Their relationship to naturally occurring thrombus formation in humans is unknown, but they do offer a model for studying thrombi that are spatially and temporally defined. Since thrombus formation following laser injury is observed over a time course of 1 to 3 minutes, high-speed digital capture of the fluorescence images with short exposure times is necessary. Using this system, we have revisited the concepts that have developed from in vitro studies about blood coagulation and thrombus formation over the past half century. Many of these models have been confirmed, but others need to be reconsidered. Some of the findings that now require major conceptual change in our understanding of thrombus formation are presented in detail.

Concept #1: Platelet Aggregation and Fibrin Generation Occur Simultaneously

One of the central tenets of thrombus formation has been the concept of primary hemostasis—mediated by platelets in the formation of a hemostatic plug—followed by secondary hemostasis, the generation of a fibrin meshwork to stabilize the platelet thrombus. However, it is now clear from in vivo studies of thrombus formation that platelet accumulation and fibrin generation occur simultaneously.[1]

Concept #2: Tissue Factor–bearing Microparticles are Important for Fibrin Generation

The late Dr. Yale Nemerson and colleagues described the presence of tissue factor in blood,[4] a surprising revelation since it had always been argued that tissue factor was extrinsic to blood (hence, the "extrinsic" pathway of blood coagulation), and that only during tissue injury did tissue factor come in contact with blood and initiate blood coagulation. We now appreciate that tissue factor circulates on certain cell-derived microparticles, and PSGL-1–expressing microparticles are delivered to the developing platelet thrombus via interaction of PSGL-1 with P-selectin on activated platelets.[5] The importance of this particular compartment of tissue factor depends upon the experimental injury model used or, in humans, the initiator of thrombus formation. For example, disruption of the vessel wall, a compartment rich in tissue factor, rapidly leads to thrombus formation, overwhelming the potential role of blood-borne tissue factor. However, with experimental laser-induced injury or in humans with severe inflammation, microparticle delivery of tissue factor plays an important role.[6] This tissue factor contributes significantly to fibrin propagation. Furthermore, at least early in thrombus formation, tissue factor delivery is via microparticles and not leukocytes.[7] Indeed, whether circulating leukocytes express tissue factor in normal blood remains controversial. A major remaining and unanswered question is: Why is blood-borne tissue factor associated with microparticles inactive until it becomes thrombus-associated? Does tissue factor need to be activated, or does it need to be concentrated within the thrombus to become functional?

Concept #3: The Tissue Factor Pathway And the Collagen Pathway are Independent Initiators of Platelet Activation

Many agonists that lead to platelet activation have been identified that potentially participate in the initial activation of platelets or that, derived from platelets upon their activation, activate additional platelets. In in vitro platelet aggregation studies, we term the latter the secondary wave of platelet aggregation. But which of these initial agonists are critical in vivo to thrombus formation? Using a laser-injury model, we have identified conditions in a live mouse under which tissue factor pathway–initiated platelet activation during thrombus formation is dominant.[8] Using this model, platelet activation is initiated by thrombin, and there is no platelet activation in the presence of thrombin inhibitors or mice lacking the platelet thrombin receptor. Furthermore, the absence of von Willebrand factor does not impede platelet activation in the tissue factor pathway. In contrast, the collagen pathway, best modeled by the disruption of the endothelium and exposure of the subendothelial matrix in the ferric chloride model, requires glycoprotein VI and von Willebrand factor for platelet activation. In human pathological conditions, it is also possible that either the collagen pathway or the tissue pathway of platelet activation dominates. For example, trauma with injury of the vessel wall surely involves the collagen pathway, whereas inflammation leading to thrombus formation may only involve the tissue factor pathway. It is also likely that both pathways may be involved under certain conditions. The presence of these two independent pathways needs to be considered when designing pharmacologic approaches to mitigating platelet activation.

Concept #4: Platelet Membranes Are Not Required for Supporting Protein Complex Formation During Thrombin Generation

One of the long-standing teachings has been that the tenase complex (factor IXa bound to factor VIIIa in the presence of calcium ions and membrane surfaces) and the prothrombinase complex (factor Xa bound to factor Va in the presence of calcium ions and membrane surfaces) assemble on the membrane surface of the activated platelet, and that these interactions are critical for the generation of thrombin and the development of fibrin. Although it is indeed true that activated platelets as well as many other activated cells can support thrombin generation via the exposure of phosphatidylserine on the cell membrane surface, the critical physiologically important membrane surface remains unproven. Three lines of in vivo evidence point to the fact that activated platelets are not required for fibrin generation. First, mice deficient in Par4, the mouse platelet thrombin receptor, do not form a platelet thrombus when the vessel wall is injured in the laser-injury model that is tissue factor pathway specific.[9] Yet fibrin generation in the absence of a platelet thrombus is normal. Second, the infusion of eptibatide, an $\alpha_{IIb}\beta_3$ inhibitor, into a mouse prevents platelet accumulation (Jasuja, Cho, Furie and Furie, unpublished). However, fibrin generation in the absence of aggregated platelets is normal. Third, mice genetically deficient in the β_3 integrin subunit do not make a platelet thrombus in the laser-injury model. Yet these mice do generate a normal fibrin clot. Membrane structures are

certainly required for thrombin generation. It remains to be determined whether endothelial cell membranes or microparticles can generate the membrane surfaces necessary for assembly of the tenase and prothrombinase complexes. Although platelet membranes are not required for fibrin generation, platelets themselves play a critical role in the hemostatic process.

Concept #5: Thiol Isomerases Are Required for the Initiation of Thrombus Formation

Protein disulfide isomerase, an endoplasmic reticulum-resident enzyme involved in disulfide bond formation, is known to have an extracellular presence. In vitro studies of platelets have previously demonstrated that this enzyme is secreted by platelets during their activation.[10] Furthermore, inhibition or disruption of this enzyme interferes with various platelet functions.[11-13] However, the physiologic function of protein disulfide isomerase in thrombus formation was only recently realized when experiments performed in a live mouse revealed that this thiol isomerase is required for thrombus formation.[14] Following the initiation of thrombus formation either with laser-induced injury or ferric chloride, protein disulfide isomerase (PDI) appears within and around the developing thrombus. PDI appears to be derived from endothelial cell activation and from platelet activation.[15] Through a mechanism yet to be revealed, this PDI remains associated with the developing thrombus. Inhibition of PDI with either bacitracin or a blocking monoclonal antibody completely inhibits fibrin generation and platelet aggregation.

Why is this important? Nature has designed a very complex system to segregate components required to initiate platelet activation and thrombin generation. Furthermore, nature has stored each of these components, whether enzymes, cofactors, cells, or structural proteins, in their biologically inactive form; that is, as zymogens, procofactors, resting cells, fibrinogen. Might both tissue factor and platelet receptors need to be activated before they can participate in hemostasis? This would be an elegant approach to regulating, at the initiation step, the generation of thrombi. The literature is replete with discussion of inactive or encrypted tissue factor,[16] although the molecular basis remains uncertain. Similarly, integrins such as $\alpha_{IIb}\beta_3$ have been shown to undergo conformational changes during their activation. One hypothesis that has been put forth is that these proteins undergo structural transitions based upon oxidation or reduction of allosteric disulfide bonds.[17] This concept, yet to be proven physiologically relevant, is nonetheless intriguing in that it unites the requirement for protein disulfide isomerase and thrombus formation. One can speculate that an electron transfer mechanism involving thiol isomerases initiates the near-simultaneous activation of the thrombus components only when they are in immediate proximity of each other. Fibrin generation, independent of the activated platelets, is inhibited in vivo in the absence of PDI. This strongly implicates PDI in tissue factor regulation, although the molecular details remain elusive.

In summary, the work from our group[18] and others[19-22] where we study thrombus formation in experimental animals has permitted improvements in understanding of the processes that are physiologically relevant. Nonetheless, in vitro studies of this system using biochemical and cell biological methodologies continue to be critical to understanding of thrombosis. In vivo experiments in whole animals and in vitro experiments with isolated cells and proteins are complementary approaches important for moving the field forward.

Disclosures

Conflict-of-interest disclosure: The author declares no competing financial interests
Off-label drug use: None disclosed.

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